Protective effect of abscisic Acid in a spinal cord injury model mediated by suppressed neuroinflammation

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ABSTRACT

Abscisic acid (ABA) is a phytohormone with modulatory roles. The anti-inflammatory effect of this hormone has been reported on different animal tissues. Immediately after spinal cord injury (SCI), neuroinflammation causes neuropathic pain and locomotor impairments. We investigated the impacts of ABA as an anti-inflammatory substance on an acute SCI model. The weight-drop contusion injury model was applied for inducing SCI in rats. The solvent, ABA (10, 15 μg/rat, IT), and MP (30 mg/kg, IP) were administered after injury. For the evaluation of proinflammatory gene expression, a real-time polymerase chain reaction was applied for the two inflammation markers tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). Moreover, the tail-flick and Basso, Beattie, Bresnahan (BBB) tests were performed to determine the effects of ABA on the neuropathic pain and locomotor function in the chronic phase of injury, respectively. Our data showed that ABA reduced the gene expression of TNF-α and IL-1β in the spinal cord of injured rats. It also increased the latency response to nociceptive thermal stimuli and improved locomotor function. Our findings showed the anti-inflammatory impacts of ABA in improving neuropathic pain and locomotor functional recovery after SCI.

Abbreviations

SCI: Spinal cord injury
CNS: Central nervous system
ABA: Abscisic acid
CSF: Cerebrospinal fluid

MP: Methylprednisolone
IL-1β: Interleukin-1β
TNF-α: Tumor necrosis factor
BBB test: Basso, Beattie, Bresnahan test

Introduction

Spinal cord injury (SCI) is a debilitating disease that leads to long-life physical impairment [1]. The last statistics in 2020 showed that approximately 54 cases per million people in the United States were affected by SCI [2]. The pathological events following SCI are divided into two broad events of primary and secondary injuries [3]. Primary injury is mechanical damage to the spinal cord and elicits a series of pathophysiological cascades that are termed secondary injuries [4]. The secondary insults exacerbate injury to the spinal cord because of leading to the destruction of axonal tracts and being one of the main barriers against functional recovery after SCI [5]. Neuroinflammation in the lesion area is the main biological event in secondary injuries [6]. Inflammation is one of the causes of neural damage after SCI [7]). Neuropathic pain is one of the other consequences of neuroinflammation, which occurs in the chronic phase of CNS injuries. Neuropathic pain is mediated by the neuroinflammatory response and follows pathological changes after injury. The role of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 is clarified by their pathological effects on neuropathic pain in the CNS [8, 9]. TNF-α has a critical role in recruiting other immune cells to participate in neurodegeneration procedures. It seems that preventing TNF-α upregulation can reduce pain and the progress of Wallerian degeneration [9]. The spinal cord has a more pronounced inflammatory response to injury than the brain, with twice as much neutrophil infiltration within 24 h, sustained macrophage infiltration, and enhanced lymphocyte infiltration [10]. This property makes the spinal cord vulnerable to secondary lesions [11]. Some studies demonstrated that the prevention of proinflammatory cytokines, including IL-1β and TNF-α, markedly improved functional recovery and induced injury apoptosis after SCI [11-13]. Therefore, inflammation limitation after SCI can be considered an important therapeutic target. Out of many chemical and natural agents with anti-inflammatory effects, MP is used widely [14].

Unfortunately, the role of steroids such as MP in acute SCI (ASCI) is unclear, and it has been correlated with an increased risk of pneumonia and hyperglycemia [15]. ABA presents in vegetables and fruits and can be obtained naturally through food. It is also generated endogenously in some human and animal tis-

sues. Many studies have shown that ABA benefits human health. ABA has antidiabetic [16], antioxidant, and antiapoptotic [17] properties. It is also effective in curing ischemic retinopathy because of its angiogenic properties [18]. Another property of APA is its anti-inflammatory role in the CNS and other tissues [19-21]. ABA has no side effects even in high doses [22]. Therefore, we investigated the effect of ABA treatment on neuropathic pain and functional motor recovery in the chronic phase of SCI. Furthermore, molecular assessments were applied for evaluating the results of preventing inflammation in the recovery of insult.

Results

Effects of abscisic acid on TNF-α and IL-1β gene expression

As shown in Figure 1, in comparison with the control group, TNF-α was significantly upregulated in SCI and SCI + solvent injected groups ($p < 0.001$). Compared to SCI and SCI + solvent injected groups, the expression of TNF-α significantly reduced in the ABA and MP-treated groups ($p < 0.001$) (Figure 1A). IL-1β expression was higher in the SCI, SCI + solvent ($p < 0.001$), and SCI + ABA (10 μg/rat) groups ($p < 0.01$) in comparison with the control group. The expression of IL-1β significantly decreased in the SCI + ABA (10 μg/rat) ($p < 0.01$), SCI + ABA (15 μg/rat), and MP-treated groups compared to the SCI and SCI + solvent groups ($p < 0.001$). All melting curves obtained from PCR light cycler Roche are shown in Figure 2 (A, B, C).

Tail-flick test

As shown in Figure 3, the tail flick nociceptive threshold was significantly lower in the SCI and all SCI-treated groups when compared with the control group ($p < 0.001$). This parameter showed a significant increase from day 1 to 30 in the SCI + ABA (10, 15 μg/rat) and SCI+MP-treated groups in comparison with both SCI and SCI + solvent groups ($p < 0.01$ and $p < 0.001$). The thermal threshold was significantly higher in the SCI + MP-treated group in comparison with SCI + ABA (10 μg/rat) injected rats after 20 days ($p < 0.001$). Except on day 5 ($p < 0.01$), there was no significant difference between the SCI + MP and SCI + ABA (15 g/rat) groups. The tail flick nociceptive threshold showed a significant rise from day 5 to 30 in SCI + ABA (15 μg/rat) treated animals in comparison with SCI + ABA (10 μg/rat)-treated group ($p < 0.001$ and $p < 0.01$).

BBB test

Locomotor functional recovery after treatment

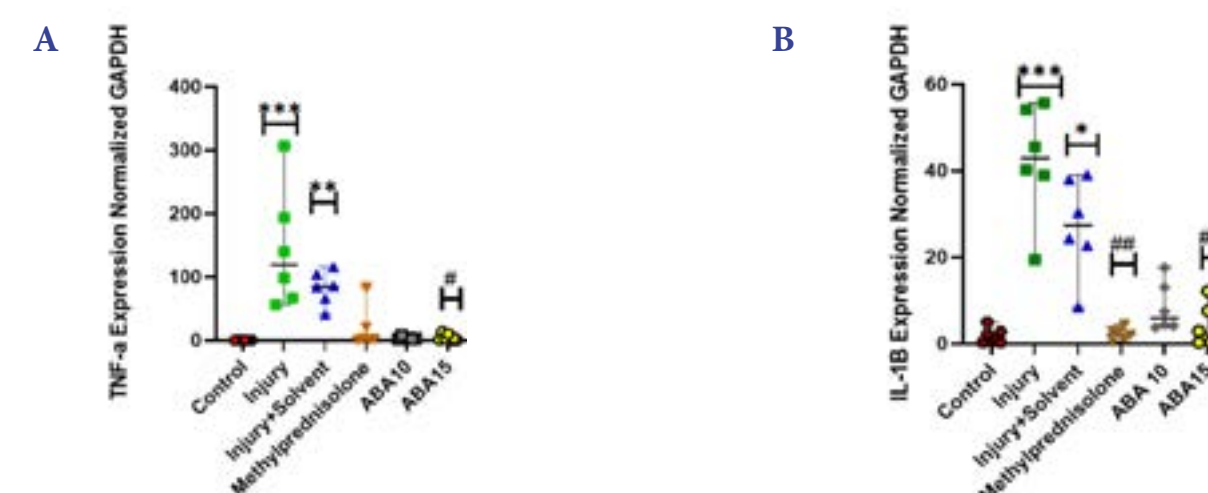**Figure 1.**
The effects of abscisic acid (ABA) on gene expression of the proinflammatory markers TNF-α (A) and IL-1β (B) in the acute phase of SCI. Reported *p* values on graphs represent the statistical testing with ANOVA and Tukey's post hoc test. **: $p < 0.01$ and ***: $p < 0.001$ indicate the significant differences in comparison with the control group. ##: $p < 0.01$ and ###: $p < 0.001$ show the significant differences versus SCI and SCI with solvent injected groups.

by ABA was assessed with the BBB test. Our results showed a remarkable rise in BBB scores in the control group compared to all SCI-induced groups ($p < 0.001$). The BBB scores were significantly higher in the SCI+ABA (15 μg/rat)-treated rats during days 3-30 in comparison with the SCI and SC + solvent injected groups ($p < 0.01$ and $p < 0.001$). As presented in Figure 4, the same effect was seen in SCI + ABA (10 μg/rat)-treated rats on day 3 and from day 13 to 30 ($p < 0.05$, $p < 0.01$, and $p < 0.001$). The BBB scores in the SCI + MP-treated group showed a significant increase from day 11 to 30 ($p < 0.01$ and $p < 0.001$).

There was a significant difference between SCI + ABA (15 μg/rat) and SCI + MP-treated groups from day 7 to 9 ($p < 0.05$). A significant difference was found between SCI + ABA (10 μg/rat) and SCI + MP-treated groups on day 19 and during days 25-30 ($p < 0.05$ and $p < 0.01$). The BBB scores showed a significant increase from day 7 to 9 ($p < 0.01$), and on day 17 ($p < 0.001$) in SCI + ABA (15 μg/rat)-treated group in comparison with SCI + ABA (10 μg/rat)-treated group (Figure 4).

Discussion

The results of the present study indicated that the IT administration of ABA immediately after SCI inhibited proinflammatory cytokines TNF-α and IL-1β and subsequently improved neuropathic pain and locomotor function.

The damage after SCI triggers inflammatory responses with enhanced TNF-α, Il-1β, and other proinflammatory marker levels [23]. This process started by the blood-brain barrier damage and neural
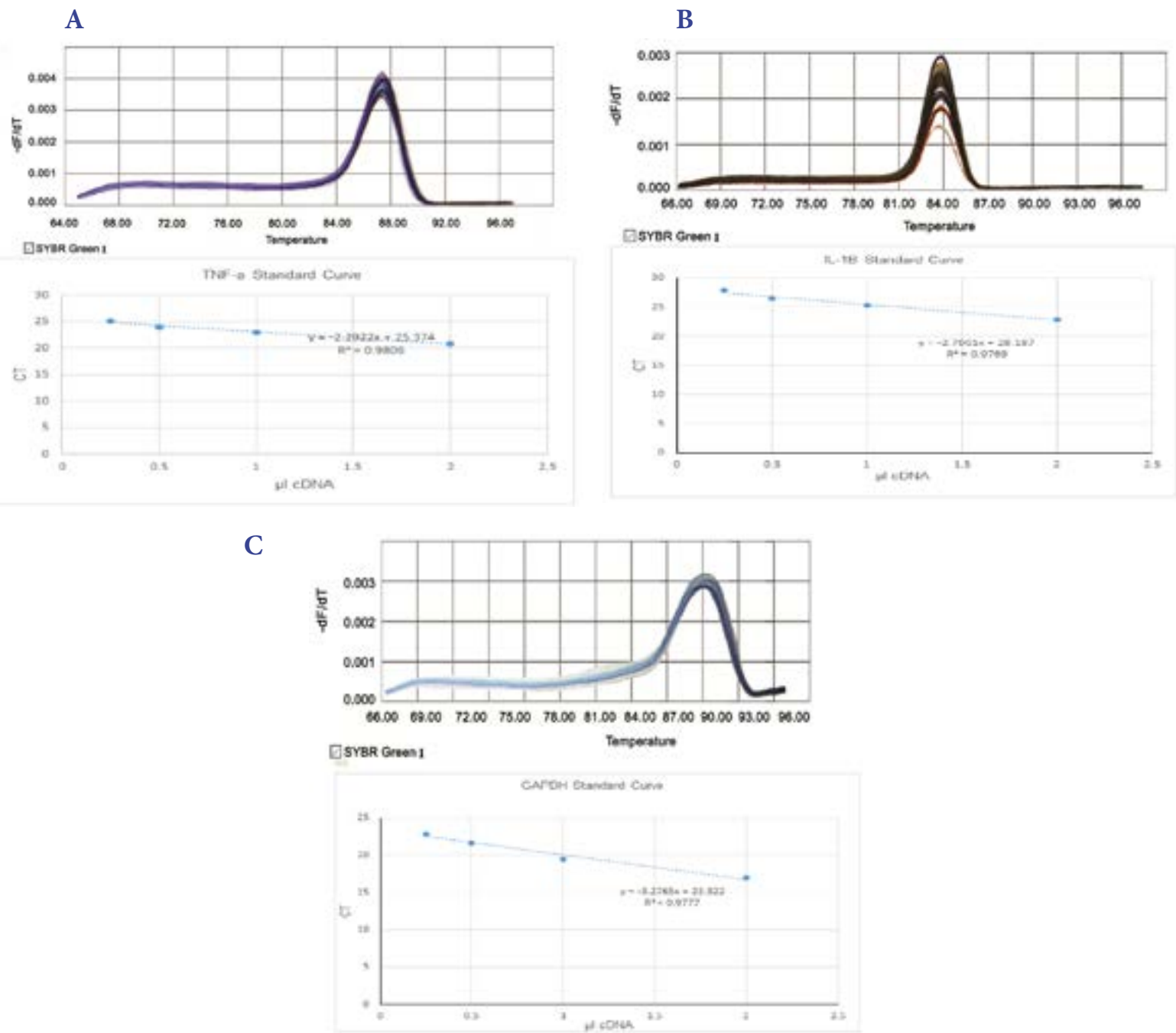
insult causes the early expression of cytokines in the injured spinal cord models in humans and animals. Just 30 min after SCI, both TNF-α and IL-1β levels raised and remained at the peak levels for 6 h. Different cells in the CNS tissues, such as astrocytes and microglia cells, immediately express these inflammatory mediators [24]. The molecular results of this study confirm the anti-inflammatory role of ABA. In line with this study, other investigations showed the anti-inflammatory effect of ABA in the CNS and other tissues [25-27]. There is an interaction between the immune system and the sensory nervous system in the generation of neuropathic pain. In other words, neuropathic pain is the result of somatosensory lesions that induce extreme inflammatory responses [28]. The results of the tail flick test in the current study indicated that the administration of ABA had antinociceptive effects and rose the latency response to thermal stimuli. Moreover, the BBB test showed an improvement in locomotor function following ABA administration. Our findings are in line with those of Mollashahi et al. (2018) who discovered that ABA had antinociceptive results in animal models for nociceptive tests, including hot plate, tail flick, and formalin tests. Furthermore, ABA was shown to impose its antinociception effects via the PPAR β/δ and opioid receptors [29]. Guri et al. demonstrated that human ABA-related genes are anchored to PPARγ with four homologs. In obese mice, ABA reduced the inflammation activated by microglia and white fat tissue-infiltrated macrophages. Nuclear receptors of PPARγ were activated by ABA in pre-adipocyte cell culture [25, 30].

The PPARs, which are defined as nuclear hormone receptors, are expressed in different sites of the CNS

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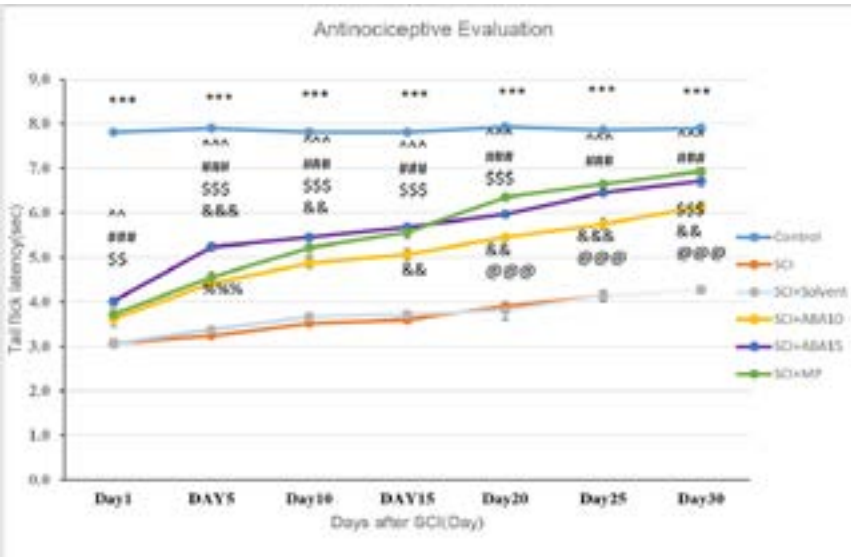DOI: 10.22067/ijvst.2022.75655.1127

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A

B

C

Figure 2.
The melting and standard curves.
A: TNF-α melting curve and standard curve. B: IL-1β melting curve and standard curve.
C: GAPDH melting curve and standard curve.

Figure 3.
The assessment of antinociceptive effect of abscisic acid (10, 15μg/rat) and methylprednisolone in the tail flick test during 30 days. Values are shown with mean ± S.E.M (n = 6 rats/group). ***$p < 0.001$ shows a significant difference in comparison with all SCI-induced groups. ### $p < 0.001$ shows the significant difference between SCI+ABA (15μg/rat) group and SCI and SC+ solvent injected groups. $$p < 0.01$ and $$$ $p < 0.001$show significant differences between SCI+MP and SCI and SC+ solvent injected groups. ^^ $p < 0.01$ and ^^^$p < 0.001$show significant differences between SCI+ABA (10μg/rat) group and both SCI and SC+ solvent injected groups. %%%$p < 0.001$shows the significant difference compared to SCI+ABA (15μg/rat) and SCI+MP group. @@@$p < 0.001$shows

the significant difference between the SCI+MP group and SCI+ABA (10μg/rat) group. && $p < 0.01$ and &&&$p < 0.001$show the significant difference in comparison between SCI+ABA (15μg/rat) and SCI+ABA group (10μg/rat) group. ABA10: abscisic acid-treated (10μg/rat), ABA15: abscisic acid-treated (15μg/rat), MP: methylprednisolone.

Figure 4.
The assessment of abscisic acid (10, 15μg/rat) and methylprednisolone effects on locomotor function via the BBB test during 30 days. Values are shown with mean ± S.E.M (n = 6 rats/group). ***$p < 0.001$ shows a significant difference compared to all SCI-induced groups. ## $p < 0.01$ and ### $p < 0.001$ show the significant difference between SCI+ABA (15μg/rat) group and both SCI and SCI+ solvent injected groups. ^$p < 0.05$, ^^ $p < 0.01$, and ^^^ $p < 0.001$ show the significant differences when comparing the SCI+ABA (10μg/rat) group with both SCI and SCI+ solvent injected groups. $$ $p < 0.01$ and $$$ $p < 0.001$ show the significant difference in comparing the SCI+MP group with SCI and SC+ solvent injected group. %$p < 0.05$ shows a signifi-

cant difference when comparing SCI+ABA (15μg/rat) group with the SCI+MP group. @$p < 0.05$ and @@$p < 0.01$ indicate the significant differences between ABA+SCI (10μg/rat) and the SCI+MP groups. && $p < 0.01$ and &&& $p < 0.001$ show significant differences between ABA+SCI (15μg/rat) and ABA+SCI (10μg/rat) groups. ABA10: abscisic acid-treated (10μg/rat), ABA15: abscisic acid-treated (15μg/rat), MP: methylprednisolone.

Figure 5.
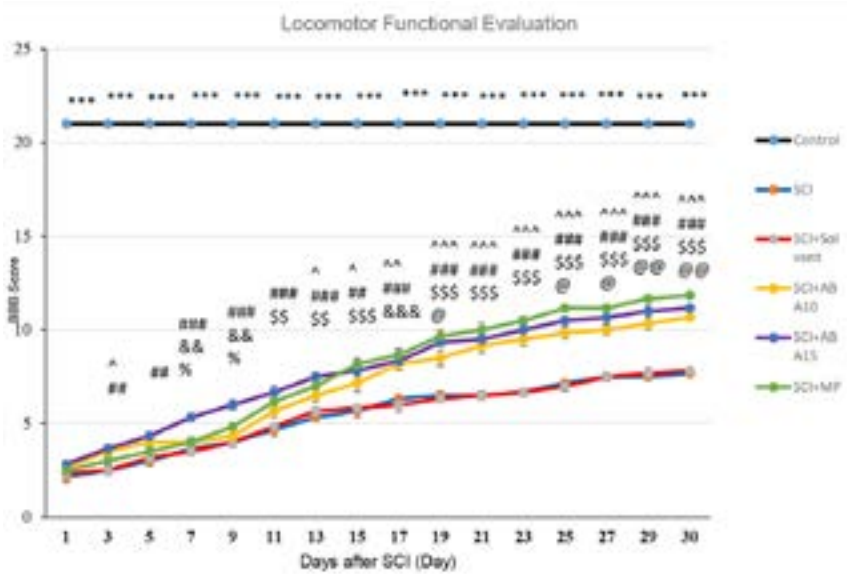The procedure of molecular and behavioral experiments.

related to pain modulation. These areas are composed of the thoracic and lumbar spinal cord, rostral ventromedial medulla, amygdala, and periaqueductal grey [31, 32]. All three isotypes of PPARs, β/δ, and γ, can modulate the inflammatory response and nociceptive reactions after SCI [33]. The result indicates the neuroprotective effects of PPAR agonists in the model of SCI, although their specific roles are not entirely understood based on their isotype [33]. The results of some studies indicated that the neurogenic inflam-

mation mechanism possibly is modulated by PPARs agonists by preventing substance P and histamine release from dorsal root ganglion. Moreover, the role of PPARs in pain modulation confirmed by molecular and pharmacological investigations [34], and its peripheral anti-inflammation effects have been reported [35]. The agonists of PPAR-γ ameliorate functional motor recovery via reducing gliosis and apoptosis, as well as preventing inflammatory mediators in the rat model of SCI approved by the FDA [36-38]. It is sug-
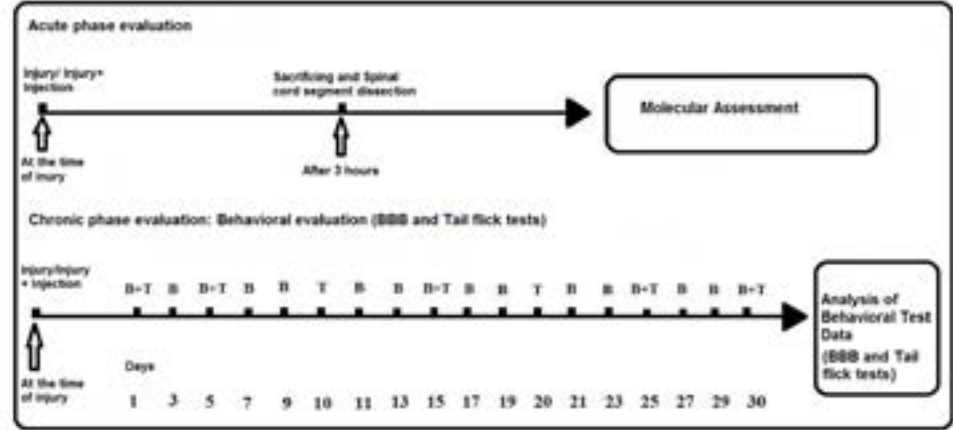
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gested that PPARγ agonist has anti-inflammatory potential and can cooperate in neuroprotective actions in CNS injuries [39, 40]. ABA structurally resembles thiazolidinediones which is the PPAR-γ agonist and may control neuroinflammation by decreasing the TNF-α level [25, 41].

Recently, it was shown that the IT administration of ABA enhanced the nociceptive threshold in the tail flick and hot plate tests. Indeed, the pharmacological suppression of protein kinase A prevented these effects [42]. These studies showed that ABA imposed its beneficial effects on reducing neuropathic pain and improving recovery after SCI via PPAR and PKA signaling pathways.

Overall, the present study indicated that ABA causes a significant reduction in the expression of proinflammatory genes TNF-α and IL-1β in the spinal cord of injured rats. Behavioral data in line with molecular findings demonstrated that ABA increases the latency response to nociceptive thermal stimuli and improves locomotor function. The findings of this research confirm the potential role of ABA as an anti-inflammatory and antinociceptive after SCI. However, more detailed studies are required.

Materials and Methods

Animals

Male Wistar rats (n = 108, W = 220-250 gr) were procured from the animal house of Shahid Bahonar University of Kerman. Animals had ad libitum access to food and water. They were kept under a 12 h light/dark cycle at 22°C ± 2°C temperature and were randomly divided into two groups for molecular and behavioral assessments. All tests were approved by the Ethics Committee of Shahid Bahonar University of Kerman (Approval No. IR.KMU. REC. 1399. 096), which follows the ARRIVE guidelines [43]. Moreover, standard ethical guidelines were considered for assessing pain in animals [44].

Medications

The (±)-cis trans-ABA was acquired from Sigma-Aldrich Co. (USA). It was diluted with artificial CSF (aCSF) after dissolving in DMSO. ABA was injected into the spine after inducing SCI by two different dosages: ABA10 (10 μg/rat, IT), and ABA15 (15 μg/ rat, IT). The solvent was also administered IT (aCSF + DMSO at the ratio of 2:1 v/v). MP sodium succinate (30 mg/kg, IP) was procured from Exir Pharmaceutical Co. (Iran).

Experimental design

Rats were randomly divided into six experimental groups (n = 6). The control group did not receive any surgery or treatment and in the SCI group, just SCI was induced without any treatment. The SCI + solvent-treated group received ABA vehicle (aCSF + DMSO) and SCI + ABA-treated groups received different doses of ABA (10 or 15 μg/rat) administrated IT and the SCI + MP-treated group was administered 30 mg/kg of MP IP. All IT injection volumes were 5 μl, and MP injected volume was 0.5 ml. Figure 5 shows the procedure of this study.

Spinal cord injury induction

Ketamine and xylazine (60 and 10 mg/kg IP, respectively) were applied to anesthetize the animals. Next, the rats were fixed in stereotaxic apparatus during the surgery. After shaving the skin and completing antiseptic procedures with 7.5% povidone-iodine, fascia and paravertebral muscles were gently dissected to expose the lamina. Dorsal laminectomy was performed without insulting the dura matter at T9-T10 to expose the spinal cord. In order to induce SCI, we used the weight-drop contusion injury model [45]. Briefly, it was performed by dropping 10 gr weight from a height of 25 mm above the exposed spinal cord [46]. Then injections were completed according to the study design. In the end, the muscle and fascia layers were sutured with absorbable sutures, and animals were transferred to a cage with a circulating heating pad for recovery.

The animals were anesthetized and sacrificed 3 h after SCI and injections. For the molecular study, intracardial perfusion was performed by phosphate buffer (pH = 7.35) and after tissue sampling, the specimens were stored in a -80°C freezer. For evaluating locomotor functions and pain response in the chronic phase, all SCI-induced groups received gentamicin for up to 5 days (12 mg/kg/day, IM) after the operation to diminish the infection rate. In addition, the rats received bladder massage three times a day to evacuate the bladder until the micturition reflex was re-established.

Tail flick test

The tail flick analgesia test estimates the analgesic properties of pharmacological substances at spinal and supraspinal levels. The tests began on the first day after the injury and continued every day until day 30. In this apparatus, the central analgesic effect of ABA was determined via the radiant heat algesimeter (Hugo Sachs Electronic, Germany). The last third of the tail was placed on a heat source to determine the analgesic reaction time (delay to tail withdrawal). Baseline threshold and deviation from the baseline due to treatments were recorded. Finally, the mean value of three measurements was applied for analysis. We considered 10 sec as the cut-off time to prevent the irritation of the animal's tail.

BBB tests

To assess the effect of the acute administration of ABA on functional recovery after SCI, we recorded the behavioral BBB test, which monitors each rat's movement in an open-field area for 5 min. The range of BBB score is from 0 (without any movement in a hind limb) to 21 (normal motion with the same interval steps), defined in three stages early, intermediate, and late.

In the early stage, scores are 0-7, defined as no hindlimb movement or isolation in motion in hindlimb joints. The intermediate stage scores are 8-13, implying proportion movement between the hindlimb and forelimb without solidarity in steps interval. The last stage score of 14-21 indicates coordination between the forelimb and hindlimb, return of toe and tail position, and trunk constancy during stepping. The test was performed a day after injury and continued every 2 days until 30 days after injury.

Molecular assay

Tissue isolation, RNA extraction, and reverse transcription

Total RNA from all spinal cord tissues of all groups was extracted with the Wizbio Reagent Master Kit protocol (Wizbio, South Korea), and the NanoDrop spectrophotometer (Thermo Scientific, USA) was used to verify the RNA isolation method and

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Table 1.
Primer sequences, RT-PCR fragment lengths, and NCBI accession numbers.

Primer name	Primer sequence	PCR amplicon	NCBI accession number	Pre-denaturation Temperature/time (s)	Denaturation Temperature/time (s)	Annealing Temperature/time (s)	Extension Temperature/time (s)	cycles
GAPDH	F: GTCTTCACCACCACGGAGAAGGC R: ATGCCAGTGAGCTTCCCGTTCAGC	392	NM_017008.4	95/300	95/20	60/30	72/20	40
TNF-α	F: ACCAGCAGATGGGCTGTACCTTAT R: ATGAAATGGCAAATCGGCTGACGG	107	NM_012675.3	95/300	95/20	60/30	72/20	40
IL-1β	F: AAGACACGGGTTCCATGGTGAAGT R: TGGTACATCAGCACCTCTCAAGCA	97	NM_031512.2	95/300	95/20	60/30	72/20	40

evaluate the concentration of RNA. We applied 1 ml Wizol Reagent for homogenizing 50 mg of spinal cord tissue. Afterwards, 200 μl of chloroform was added to each tube for RNA isolation. The washing stage was completed with 500 ml of isopropanol and 75% ethanol. The tubes were centrifuged after adding the chemical solution for isolating RNA from tissue samples and ultimately, a proper volume of RNase-free water was added to each tube. A volume of 10 μl of RT master mix which contained MMLV RTase (Wizbio, South Korea) and 1 μl oligo (dT) were added to 1 μg RNA of each template. Next, each sample volume was increased to 20 μl with RNase-free water.

Real-time PCR

The assessments were improved by applying Bio-Rad PCR iQ5 Thermal Cyclers (Bio-Rad, Richmond, CA, USA) to synthesize the first strand of cDNA. Next, 1 μl forward and 1 μl reverse primers and qPCR master mix were added and prepared for PCR with 10 μl SYBR green reporter dye. The Real Q Plus 2X master mix (Bio FACT, South Korea) was used in the PCR reactions and the final volume reaction in each tube was 20 μl. Samples were performed in triplicates on Roche PCR light cycler (Roche Life Science, Germany), and its software version 1.1 was utilized for analyzing the gene expression data. The fluorescence melting curves were generated to screen the primer dimers. For predicting all the sizes of primer, the PCR products were run on the electrophoresis 1.5% agarose gel (Sigma) with DNA loading dye (Smobio, Taiwan) and then using Ingenius 3 Gel Documentation (Syngene Bio-Imaging, UK) for visualization. Samples were normalized with the housekeeping gene GAPDH, as well as TNF-α and IL-1β genes as the proinflammatory markers (47). We evaluated PCR efficiency and linearity using four points of diluting and over twofold concentration of cDNA which obtained 0.9769, 0.9806, and 0.9777 for IL-1β, TNF-α, and GAPDH, respectively (Figures 2A, 2B, 2C). All the information on the primers, including their sequences, size of PCR product, NCBI accession numbers, and PCR stages based on temperature and time(s) are mentioned in Table 1 (48). The PCR efficiency for the primers was not 1. As a result, we applied $1.96^{-\Delta\Delta CT}$ for TNF-α and $1.95^{-\Delta\Delta CT}$ to determine the relative gene expression ratio.

Statistical analysis

Molecular and behavioral datasets were analyzed by the SPSS software. The one-way analysis of variance with Tukey's posthoc test was applied to assess the differences between the study groups. The mean ± SEM was presented for all data, and values of $p < 0.05$ were considered statistically significant.

Authors' Conributions

All authors conceived and planned the experiments. Maryam Rezaeezadeh Roukerd carried out the experiments and analyzed data set. Mehdi Abbasnejad contributed to the interpretation of the results. Sahel Motaghi took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Acknowledgements

Conflicts of Interest

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